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Understanding the Complex Pathobiology of High Pathogenicity Avian Influenza Viruses in Birds

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SUMMARY. Avian influenza (AI) viruses are a diverse group of viruses that can be divided into 144 subtypes, based on different combinations of the 16 hemagglutinin and nine neuraminidase subtypes, and two pathotypes (low and high pathogenicity [HP]), based on lethality for the major poultry species, the chicken. However, other criteria are important in understanding the complex biology of AI viruses, including host adaptation, transmissibility, infectivity, tissue tropism, and lesion, and disease production. Overall, such pathobiological features vary with host species and virus strain. Experimentally, HPAI viruses typically produce a similar severe, systemic disease with high mortality in chickens and other gallinaceous birds. However, these same viruses usually produce no clinical signs of infection or only mild disease in domestic ducks and wild birds. Over the past decade, the emergent HPAI viruses have shifted to increased virulence for chickens as evident by shorter mean death times and a greater propensity for massive disseminated replication in vascular endothelial cells. Importantly, the Asian H5N1 HPAI viruses have changed from producing inconsistent respiratory infections in 2-wk-old domestic ducks to some strains being highly lethal in ducks with virus in multiple internal organs and brain. However, the high lethality for ducks is inversely related to age, unlike these viruses in gallinaceous poultry, which are highly lethal irrespective of the host age. The most recent Asian H5N1 HPAI viruses have infected some wild birds, producing systemic infections and death. Across all bird species, the ability to produce severe disease and death is associated with high virus replication titers in the host, especially in specific tissues such as brain and heart.

RESUMEN. Entendiendo la compleja patobiología de los virus de influenza aviar de alta patogenicidad en aves.

Los virus de influenza aviar comprenden un grupo de virus que pueden dividirse en 144 subtipos basados en las diferentes combinaciones de los 16 subtipos de hemoaglutininas y nueve neuraminidasas, con dos patotipos, alta y baja patogenicidad, basado en la letalidad para las aves domésticas. Sin embargo, para entender la compleja biología de los virus de influenza aviar, existen otros criterios importantes como la adaptación al huésped, la transmisión, la infectividad, el tropismo tisular, las lesiones y la capacidad de producir la enfermedad. Estas características patobiológicas varían de acuerdo con la especie afectada y la cepa viral. Experimentalmente, los virus de influenza aviar de alta patogenicidad típicamente producen una grave enfermedad sistémica con alta mortalidad en las aves domésticas y otro tipo de gallináceas. Sin embargo, estos mismos virus usualmente no producen signos clínicos de infección o solamente una enfermedad leve en patos domésticos y otras aves salvajes. Durante la última década, los virus de influenza aviar de alta patogenicidad que han aparecido se han caracterizado por un incremento en su virulencia para las aves domésticas, como se puede observar por sus tiempos promedio de mortalidad más cortos y una mayor inclinación a diseminarse masivamente en las células endoteliales del sistema vascular. Por ejemplo, los virus Asiáticos de influenza aviar H5N1 de alta patogenicidad han cambiado, encontrándose cepas que producen infecciones respiratorias incoherentes en patos domésticos de dos semanas de edad, hasta cepas que son altamente letales en patos, con la presencia de virus en varios órganos internos y en el cerebro. Sin embargo, la alta letalidad para los patos está inversamente relacionada con la edad, a diferencia de estos virus en otras especies aviares, que son altamente patógenos a cualquier edad. Los virus Asiáticos de influenza aviar H5N1 de alta patogenicidad encontrados más recientemente han infectado algunas aves salvajes, produciendo infecciones sistémicas y mortalidad. En todas las especies aviares, la capacidad de producir enfermedad severa y mortalidad está asociada con los altos títulos de multiplicación viral en el huésped, especialmente en tejidos específicos como el cerebro y el corazón.

Key words: avian influenza, avian influenza virus, influenza, pathobiology

Abbreviations: AI = avian influenza; DPI = days postinoculation; EID_{50} = mean embryo infectious dose; H or HA = hemagglutinin; HP = high pathogenicity; HPAI = high pathogenicity avian influenza; IN = intranasally; IV = intravenously; LP = low pathogenicity; LPAI = low pathogenicity avian influenza; MDT = mean death time; N or NA = neuraminidase

Avian influenza (AI) viruses are a diverse group of viruses in the family Orthomyxoviridae, genus *Influenzavirus A* and can be categorized into subtypes based on the two surface glycoproteins, the hemagglutinin (H) and neuraminidase (N) (38). There are 16 different hemagglutinin (H1–16) and nine different neuraminidase (N1–9) subtypes, which make 144 possible combinations of H and N subtypes (10). Avian influenza viruses can be further classified into two different pathotypes (low [LP] and high pathogenicity [HP]), based on the ability to produce disease and death in the major domestic poultry species, the chicken (*Gallus domesticus*) (41). However, understanding additional criteria are important in comprehending the complex pathobiology and maintenance of low pathogenicity avian influenza (LPAI) and high pathogenicity avian influenza (HPAI) viruses. These criteria include exposure, host adaptation, infectivity, pathobiological changes, and transmissibility (37).

PATHOBIOLOGY CONCEPTS

First, exposure, which is access to the virus, is critical in beginning the process of avian influenza infection. The lack of virus exposure fails to produce infection such as in AI-free countries or countries that have AI viruses, but the birds are in AI-free compartments or zones. However, with some AI virus strains and some hosts, exposure to virus may not result in infections, especially if the route of exposure in inappropriate, the exposure dose is below the infection threshold, immunity against the virus strain is present, the virus strain is not adapted to the specific host species, or a combination.

Table 1. Time sequence of lesions, virus isolation, and AI viral antigen in specific pathogen free broiler chickens intranasally inoculated with 10^6 EID_{50} H5N1 HPAI virus (A/Hong Kong/156/97). Blank cells = not examined; −, negative for virus or lesions.

	Virus tissue titers (log_{10} EID_{50}/g)				
Sample time (hr)	Brain	Spleen	Cloaca	Lesions	Immunohistochemistry
1				−	−
2				−	−
4				−	−
8	(1.97)[A]	−	−	−	−
16				Mild pneumonia	Respiratory epithelium
24	3.5	5.6	1.5	Rhinitis	Respiratory epithelium > vascular endothelium
36				Systemic	Vascular endothelium, parenchyma
48	7.5	8.7	4.6	Systemic	Vascular endothelium, parenchyma

[A]Brain from one of two chickens was virus positive. All other samples were the mean titers of two positive chickens.

Second, host adaptation, which is the result of progressive genetic changes in a virus, results in increasing efficiencies of binding, replication, and release of the virus from a specific host species. Viruses with low adaptation fail to replicate in the host species unless there is high exposure dose or secondary factors that increase host susceptibility. By contrast, with a high degree of adaptation to the host species, low doses of the virus strain are needed to produce infection. Host adaptation is maximal for a single host species. Although in evolutionarily closely related species, the virus strain may show some, but a lesser degree of adaptation than the optimal host species. Third, infectivity is the ability of the virus strain to bind to cells of a specific host, replicate, and release infectious virus. There are three potential clinical outcomes with AI infection in birds: no clinical signs, mild disease, and severe disease with death. Fourth, pathobiological changes are abnormal physiological and anatomic changes that occur as a result of virus replication within the cell, tissue, organ, or a combination. In general, as virus replication titers increase, so do the severity of pathobiological changes such as gross and microscopic lesions with the most pathogenic virus strains causing major cell damage and death if it is sufficiently severe to effect critical organs. Fifth, transmissibility is natural host-to-host spread and is dependent upon an adapted virus; exposure to the virus through infected animals or fomites; and a naïve, susceptible host.

Ecology and epidemiology. LPAI viruses are maintained in wild bird reservoirs, predominately the aquatic birds in the orders Anseriformes and Charadriiformes (30,44). These viruses are passed within and between species of birds that share the same ecosystems, and such infections are typically not associated with diseases. Gallinaceous poultry (e.g., chickens, turkeys, quail, and guinea fowl) within various agricultural systems can become infected by such viruses, but this requires two steps: exposure and adaptation. Exposure can result from outdoor rearing, outdoor access for semiconfined poultry, wild bird access to buildings, or environmental exposure such as use of virus-contaminated surface water. However, this exposure will only result in significant infection if the virus is sufficiently adapted for the host species; otherwise, the virus may circulate inefficiently in the population until it adapts to the new host species and emerges as a clinically significant problem, or it may die out without becoming established in the bird population. Once adapted to gallinaceous poultry, AI viruses rarely return back to wild birds because they are now de-adapted to wild birds. These gallinaceous poultry-adapted AI virus circulate among agriculture systems whether village, semicommercial, or commercial sectors. On occasions, some of the H5 and H7 LPAI viruses have abruptly changed pathobiologically from LP to HP through changes in the proteolytic cleavage site of the H protein (23). Once this change occurs, the HPAI viruses remain adapted to gallinaceous poultry and typically do not go back into wild birds There are a few examples of isolated wild birds being infected, but establishment of lineages of HPAI viruses in wild birds has not occurred until recently (28,32,34). For example, Alexander *et al.* (1) tested six HPAI viruses isolated between 1959 and 1983 in a simulated natural exposure (intranasal) by using surrogate domestic ducks, which are the same species as the wild mallard (*Anas platyrhynchus*) (1). He observed ducks were resistant to infection or only had low-level replication and no clinical signs of disease, indicating these HPAI viruses were not adapted to mallard-type ducks. By contrast, the ecology of H5N1 HPAI virus has changed since first detected in 1997 in Hong Kong. The virus was first described in domestic geese in Guangdong, China, during 1996. followed by outbreaks in poultry in China and Hong Kong in 1997 (5,29,46). However, mortality in wild birds was first reported in Hong Kong in 2002, followed by reports in Cambodia and Thailand, and significant mortality during 2005 in wild waterfowl in Qinghai, China, and Lake Erhel, Mongolia (6,8,19). This evidence indicates the HPAI H5N1 virus has readapted back to some wild birds species and is much different ecologically and epidemiologically than previous HPAI viruses.

CRITICAL VIRUS FACTORS IN INFECTION AND VIRULENCE

The process of replication of AI viruses requires multiple steps (45). The HA must first bind to the α2,3-galactose linkage receptors on specific cells to initiate the replication cycle. By contrast, swine and human influenza A virus bind preferentially to α2,6-galactose linkage receptors, which explains why AI viruses rarely infect these species. After binding to cell receptors, the AI virus is internalized via endocytosis and the virus envelope fuses with the membrane of the endocytic vessel via a low pH-dependent conformational change of the cleaved HA of the virus. This proteolytic cleavage of the whole hemagglutinin (HA0) into the HA1 and HA2 fragments is a prerequisite for fusion. For LPAI viruses, the cleavage of the HA0 is accomplished by trypsin-like enzymes within various epithelial cells or exocellularly in respiratory secretions or by specific bacterial proteases. However, with HPAI viruses this cleavage of the HA0 is accomplished through the furin family of enzymes contained within cells of multiple tissues and organs throughout body (12). Another important viral component is the replication machinery of the AI viruses, the polymerase complex, which is necessary for use in cells to reproduce the viral nucleic acids. Finally, the neuraminidase protein is essential for virus release; and in some cases, the HA and NA

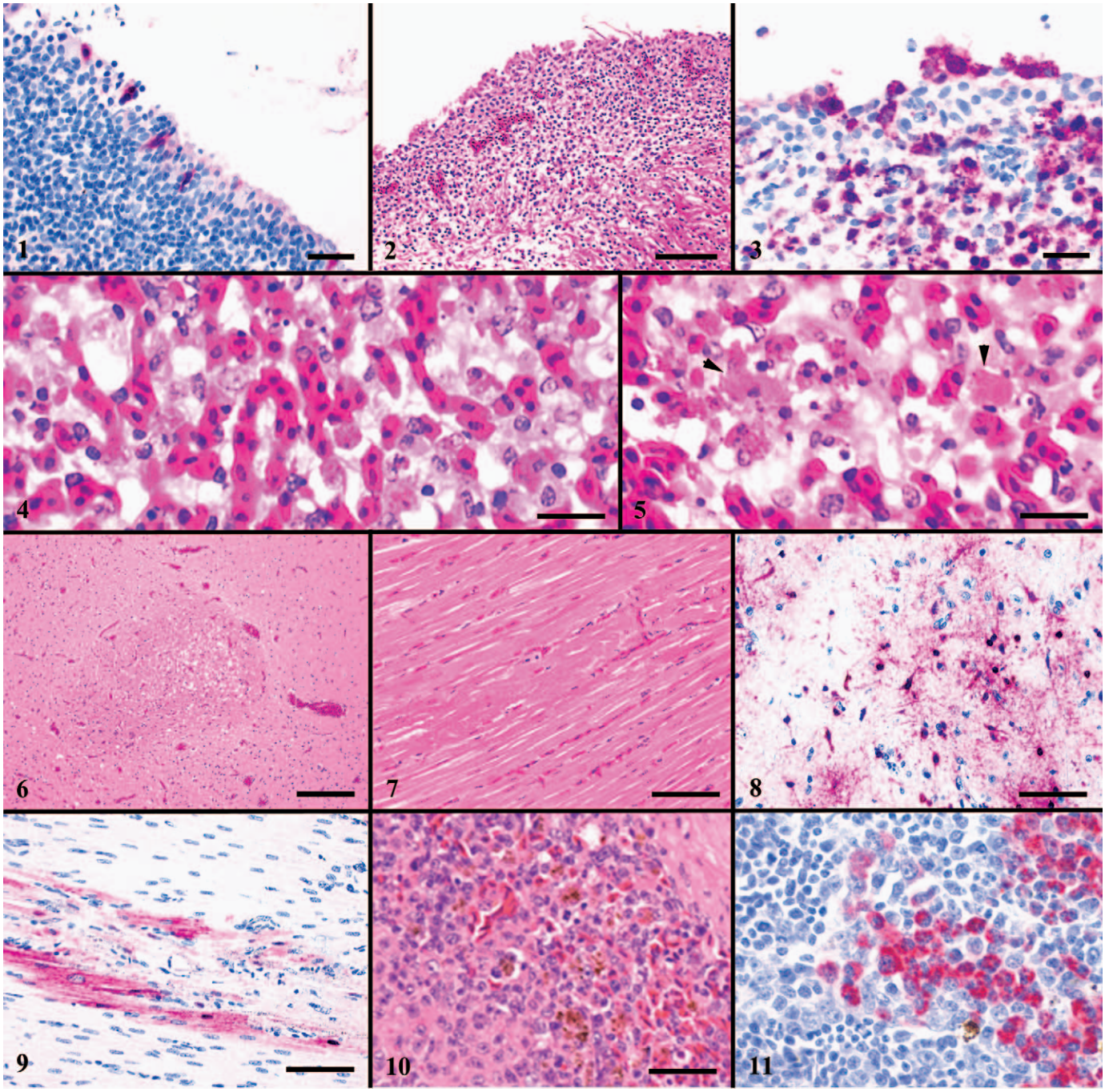

Fig. 1. Influenza A viral antigen in nasal epithelium of a chicken 16 hr after intranasal inoculation with A/whooper swan/Mongolia/244/2005 H5N1 HPAI virus. Immunohistochemical stain. Bar = 25 μm.

Fig. 2. Ulcerative rhinitis in a chicken 24 hr after intranasal inoculation with A/whooper swan/Mongolia/244/2005 H5N1 HPAI virus. HE. Bar = 75 μm.

Fig. 3. Influenza A viral antigen in nasal epithelium and underlying capillary endothelial cells and inflammatory cells of a chicken 24 hr after intranasal inoculation with A/whooper swan/Mongolia/244/2005 H5N1 HPAI virus. Immunohistochemical stain. Bar = 25 μm.

Fig. 4. Lung of chicken 24 hr after intranasal inoculation with A/whooper swan/Mongolia/244/2005 H5N1 HPAI virus, which shows hypertrophy, degeneration, necrosis, and apoptosis of endothelial cells. H&E. Bar = 25 μm.

Fig. 5. Microthrombosis (arrowheads) in lung of a chicken 24 hr after intranasal inoculation with A/whooper swan/Mongolia/244/2005 H5N1 HPAI virus. H&E. Bar = 25 μm.

Fig. 6. Severe focal necrosis in brain of a pigeon 6 days after intranasal inoculation with A/crow/Thailand/1C/2005 H5N1 HPAI virus. H&E. Bar = 200 μm.

Fig. 7. Focal myocytes degeneration and necrosis in the heart of a pigeon 6 days after intranasal inoculation with A/crow/Thailand/1C/2005 H5N1 HPAI virus. H&E. Bar = 50 μm.

Fig. 8. Abundant AI viral antigen in neurons and neuropil of the brain from a pigeon 6 days after intranasal inoculation with A/crow/Thailand/1C/2005 H5N1 HPAI virus. Immunohistochemical stain. Bar = 50 μm.

Table 2. Histological lesions in experimental infections in chickens inoculated intranasally (IN) or intravenously (IV) with A/chicken/Jalisco/14589/94 (J12/94) and A/chicken/Hidalgo/26654-1368/1994 (H5/94) Mexican H5N2 LPAI viruses and A/chicken/Pueblo/8623-607/1994 (P11/94) and A/chicken/Queratero/14588-19/1995 (Q1/95) Mexican H5N2 HPAI viruses (36) (unpublished data).

Organ	Lesion	H5/94 LP[A]	J12/94 LP[A]	P11/94 HP[A]	Q1/95 HP[A]
Heart	Myocyte necrosis	−	−	++	+++
	Myocarditis	−	−	+++	++
Brain	Neuron necrosis	−	−	+++	+++
	Meningoencephalitis	−	−	+++	+
Pancreas	Vacuolation	(+)	(++)	+++	+++
	Acinar necrosis	−	−	++	+++
Kidney	Tubule necrosis	(+++)	(++)	+	+
	Interstitial nephritis	(+++)	(+++)	+++	−
Upper respiratory tract	Rhinitis and tracheitis	[++]	[++]	++	++
Lung	Pneumonia	(+++)	(++)	+	+++

[A] − = no lesion, + = mild lesion, ++ = moderate lesion, +++ = severe lesion; ( ) = IV inoculation only, [ ] = IN inoculation only, and no ( ) or [ ] = both IN and IV inoculation.

subtypes are specifically paired to maximize replication and virus release. In summation, the proper gene constellation is essential for replication and expression of virulence by AI viruses (2).

Although, LPAI viruses can be any of the 16 HA subtypes, only some of the H5 and H7 LPAI viruses have mutated and became HPAI viruses. This change in virulence has been accomplished through several mechanisms. The first mechanism was insertion of extra basic amino acids in HA proteolytic cleavage site and substitutions of nonbasic with basic amino acids; for example, the H5N2 HPAI virus of Mexico in 1994 (11,13). The second mechanism was loss of a sugar group that covers HA cleavage area such as occurred in 1983 with H5N2 HPAI virus in United States (15). The third mechanism was insertion of abundant extraneous genetic information in the HA proteolytic cleavage site through nonhomologous recombination. The H7N3 HPAI outbreak viruses in Chile 2002 had insertion of 27 nucleotides from the nucleoprotein gene in the HA cleavage site, whereas the H7N3 HPAI virus in Canada from 2004 had insertion of 21 nucleotides from the matrix gene (22,35).

PATHOGENESIS OF HPAI VIRUS INFECTIONS IN CHICKENS

To better understand the infection process of HPAI viruses, chickens were inoculated intranasally with a HPAI virus (A/Hong Kong/156/1997 [H5N1]) and sampled at 1, 2, 4, 8, 16, 24, 36, and 48 hr after inoculation (Table 1). The AI virus was first visualized at 16 hours in respiratory epithelium of the middle nasal cavity (Fig. 1), and by 24 hr it was associated with extensive rhinitis and ulceration (Fig. 2), and extended into the underlying submucosa with viral antigen in capillary endothelial cells (Table 1; Fig. 3). In addition at 24 hr, the AI virus had spread systemically with low-to-moderate titers of virus in brain and spleen. At 48 hr, the virus titers in visceral organs were high and lesions severe with abundant viral antigen in multiple types of parenchymal cells. In the brain, a minimal level of virus was isolated from one bird at 8 hr, moderate levels from two birds at 24 hr, and high levels from two birds at 48 hr. However in looking at viral antigen, no virus was visualized in olfactory or other cranial nerves at anytime, but at 36 hr, viral antigen was restricted to capillary endothelial cells and a few surrounding microglia in the brain. At 48 hr, the viral antigen was randomly scattered throughout the brain in vascular endothelial cells, neurons, and ependymal cells. Unlike experimental studies in mice with extension from nasal epithelium via cranial nerves to the brain (42), this H5N1 HPAI virus reached the brain of chickens through vascular dissemination and invasion through brain capillary endothelium.

The pathophysiological mechanisms that are responsible for the severe illness and death with HPAI viruses vary. In some birds, altered permeability of vascular endothelial cells is responsible for the edema, hemorrhage, and multiple organ failure as evident histologically by hypertrophic, degenerating, and necrotic or apoptotic vascular endothelial cells and can be accompanied by microthrombosis (24,33) (Fig. 4). This stage can progress to become a consumptive coagulopathy with thrombocytopenia, thrombi and emboli in vessels, and activation of coagulation cascade evident by prolonged prothrombin time and high levels of tissue factor (21,24) (Fig. 5). In birds that survive the peracute phase, virus may replicate in multiple critical organs, resulting in single or multiorgan failure such as in brain and autonomic nervous system, cardiac myocytes, endocrine tissue (e.g., adrenal gland), or pancreas (27).

PATHOBIOLOGY OF HIGH PATHOGENICITY AVIAN INFLUENZA

Gallinaceous poultry. In gallinaceous domestic poultry, infection with HPAI viruses produce severe depression, severe decrease in feed and water consumption, high morbidity and

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Fig. 9. Nuclear and cytoplasmic staining for AI viral antigen in cardiac myocytes from a pigeon 6 days after intranasal inoculation with A/crow/Thailand/1C/2005 H5N1 HPAI virus. Immunohistochemical stain. Bar = 50 μm.

Fig. 10. Hemachomatosis in spleen of an American crow 9 days after intranasal inoculation with A/crow/Thailand/1C/2005 H5N1 HPAI virus. H&E. Bar = 50 μm.

Fig. 11. AI viral antigen in granulocytes and their precursors in bone marrow of an American crow 8 days after intranasal inoculation with A/crow/Thailand/1C/2005 H5N1 HPAI virus. Immunohistochemical stain. Bar = 25 μm.

Table 3. Immunohistochemical demonstration of AI viral nucleoprotein in experimental infections of chickens inoculated IN or IV with A/chicken/Jalisco/14589/94 (J12/94) and A/chicken/Hidalgo/26654-1368/1994 (H5/94) Mexican H5N2 LPAI viruses and A/chicken/Pueblo/8623-607/1994 (P11/94) and A/chicken/Queratero/14588-19/1995 (Q1/95) Mexican H5N2 HPAI viruses (36) (unpublished data).

Organ	Cell type	H5/94 LP[A]	J12/94 LP[A]	P11/94 HP[A]	Q1/95 HP[A]
Heart	Myocytes	−	−	++	+++
	Autonomic neurons	−	−	−	+
Brain	Neurons	−	−	+++	+++
	Ependyma/choroid plexus	−	−	+	+
Pancreas	Acinar epithelium	−	−	+	+++
Kidney	Tubule epithelium	(+++)	(++)	+	+
Circulatory system	Vascular endothelium	−	−	+	+
Upper respiratory tact	Epithelium	[++]	[++]	+	+

[A]AI antigen staining score: − = none, + = infrequent or rare, ++ = frequent, +++ = common; ( ) = IV inoculation only, [ ] = IN inoculation only, no ( ) or [ ] = both IN and IV inoculation.

mortality rates, sudden death, and occasionally nervous signs if they survive the peracute syndrome. However, the frequency of clinical signs and gross lesions varies with virus and species of bird and are not consistent in all birds. For example, in the H7N7 HPAI outbreak in the Netherlands, laying chickens from outbreak farms at necropsy had peritonitis (62%); tracheitis (43%); edema of the neck, wattles, or both (12%); hemorrhages in the proventriculus (4%) or no gross lesions (17%) (7). The first two lesions are commonly seen with multiple different viral and bacterial agents. In experimental studies with 1997 Hong Kong H5N1 HPAI virus, neurological dysfunction varied between turkeys (41%), partridge (28%), Bobwhite quail (14%), ring-necked pheasant (13%), and Japanese quail (8%) (24).

In chickens, the most frequently infected species with HPAI viruses, common lesions include edema to necrosis of comb and wattle, edema of the head and legs, subcutaneous hemorrhage of legs, lungs that fill with fluid and blood, and small hemorrhages on internal organs such as coronary fat. All these lesions point to alternations in the cardiovascular system, principally affecting vascular endothelium and the resulting viremia. The pathogenesis of infections by HPAI viruses has been extended by study of histological lesions and immunohistochemical localization for AI viral antigens. Experimental studies of the H5N2 Mexican LP and HPAI virus infection in chickens revealed distinctly different pathogenic mechanisms of infections (36, 40). Intranasal inoculation with two LPAI viruses produced lesions only in upper respiratory tract (Table 2) with virus localization in epithelial cells (Table 3). Intravenous inoculation produced lesions in two other epithelial organs, the kidneys and pancreas, and in the lungs. By contract, two HPAI viruses produced lesions in multiple organs with virus localization in multiple cells types irrespective of the route of inoculation. This pathogenesis and tissue tropism is a result of virus replication sites that are related to differences in enzyme cleavability of the HA protein as described above.

There can be variations in the replication and pathobiological features of an HPAI virus strain for a single species. For example, different strains of the H5N1 HPAI viruses from 1997 to 2005 caused 100% mortality in intranasally inoculated chickens and replicated to high titers in heart and brain (Table 4). However, individual strains varied in their mean death times (MDTs) from 1.5 to 5.5 days and peak replication titers in oropharynx from low ($10^{1.7}$ mean embryo infectious doses [EID_{50}]/ml) to high ($10^{7.6}$ EID_{50}/ml). H5N1 HPAI viruses isolated after 2002 tended to replicate to 10^{1-4} EID_{50} higher titer in the oropharynx than viruses from 1997 to 2001. Cloacal titers were similar irrespective of the strain.

Table 4. Differences in virus replication titers and MDTs of H5N1 HPAI viruses in intranasally inoculated 3-6-wk-old chickens (10^6 EID_{50}/bird) (modified from [39]). Blank cells = not examined, − = negative for virus.

Virus	Age (wk)	MDT	Virus titer (EID_{50}/ml or g) Oral	Cloacal	Brain	heart
Ck/HK/220/97	4	1.5	4.7	4.2	6	
Gs Env./HK/437-6/99	4	5.5	1.7	−		
Dk/Anyang/AVL-1/01	4	2.9	3.1	6.7		
Gs/Vietnam/113/01	4	2.6				
Gs/Vietnam/324/01	4	2.4				
Ck/Korea/ES/03	3–6	2	6.2	6	7.2	9.3
Ck/Indonesia/7/03	4–6	2.1	6.4	5.6	7.6	
Crow/Thailand/1C/04	4	1.8	6.8	4	6.9	9
Human/Vietnam/1203/04	4	1.5	6.3	5.9	7.7	10
Whooper swan/Mongolia/05	4	2.3	7.6	5.3	6.9	10.6

The amount of virus need to produce a lethal infection is similar for chickens with different strains of the virus when using intranasal route of inoculation. The mean chicken lethal dose for A/chicken/South Korea/2003 (H5N1) was $10^{3.1}$ EID_{50} (4). In this study, the mean chicken infection dose was the same because all the surviving chickens were AI antibody negative by using the hemagglutination inhibition and agar gel immunodiffusion tests.

Pathobiology groups. The H5N1 HPAI viruses vary in the lesions they produce depending on host species, host age, and strain of virus. The pathobiology of a prototype H5N1 HPAI virus, A/chicken/Hong Kong/220/1997, are divided into four groups (26). In group 1, the virus produced a severe systemic disease in gallinaceous birds (chickens [*Gallus domesticus*], turkeys [*Meleagris gallopavo*], Japanese quail [*Coturnix coturnix japonicus*], Bobwhite quail [*Colinus virginianus*], pearl guineafowl [*Numida meleagris*], ring-neck pheasant [*Phasianus colchicus*], and chukar partridges [*Alectoris chukar*]) and zebra finches (*Taeniopygia guttata*), with 100% morbidity and >75% mortality. Typically, the birds exhibited severe listlessness before death, and some had neurological dysfunction. Some birds may die peracutely without exhibiting clinical signs. The virus replicated in vascular endothelium and phagocytic leucocytes (chickens and turkeys) or in parenchymal cells such as in the heart, adrenal, pancreas, and brain (other gallinaceous birds and zebra finches) with accompanying necrotic and inflammatory lesions. In group 2, the virus produced severe lesions in two to three critical organs of domestic geese (*Anser anser domesticus*), emus (*Dromaius novaehollandiae*), house finches (*Carpodacus mexicanus*), and budgerigars (*Melopsittacus undulatus*). The morbidity was delayed compared with group 1, and mortality ranged from 0 to 75%. Neurological signs were common because the virus has a strong tropism for the central nervous system, although heart and pancreas also were frequently affected organs. In group 3, the virus produced in Pekin ducks (*Anas platyrhyncos*), house sparrows (*Passer domesticus*), and laughing gulls (*Larus atricilla*) minimal clinical signs, and no mortality with the infection. Lesions were limited to predominantly the respiratory tract, but to a lesser extent the heart and gonads. Virus titers in tissues with lesions were usually low. In group 4 (pigeons [*Columba livia*] and European starlings [*Sturnus*

Table 5. Mortality, MDTs, and virus replication titers from oropharyngeal (OP) and cloacal swabs, and brain of 2-wk-old domestic ducks inoculated intranasally with H5N1 (1997–2005) HPAI viruses (modified from [39]).

Virus	Mortality (no. dead/no. included)	MDT (days)	OP[A] 3 DPI	Cloacal[A] 3 DPI	Brain[A] 3 DPI
A/whooper swan/ Mongolia/244/05	7/8	4.3	5.7	5.4	8.0
A/Crow/Thailand/04	8/8	4.5	4.4	1.98	6.4
A/Egret/HK/757.2/02	7/8	4.1	5.8	2.36	5.0
A/Vietnam/1203/04	7/8	4.2	5.0	2.0	4.6
A/Prachinburi/6231/04	3/8	6.3	3.7	1.36	4.3
A/Ck/Korea/ES/03	2/8	4	1.62	41.55	ND[B]
A/Gs/Vietnam/113/01	0/8		1.8	Neg.[C]	1.5
A/Ck/HK/317.5/01	0/8		1.92	2.45	ND
A/Dk/Anyang/ALV1/01	0/8		2.5	1.3	2.1
A/Env/HK/437-6/99	0/8		2.05	2.57	Neg.
A/Ck/HK/220/97	0/8		1.97	1.22	Neg.

[A]Mean titer reported as $\log_{10}EID_{50}$.
[B]ND = no done.
[C]Neg. = virus isolation negative.

Table 6. Pathobiology group, and lesion severity and distribution for selected H5N1 HPAI viruses after IN inoculation (10^6 EID_{50}) into 2-wk-old domestic ducks.

Pathobiology group	Virus	Affected system
1	A/Egret/Hong Kong/757.2/2002, A/goose/Hong Kong/739.2/2002, A/Vietnam/1203/2004, A/crow/Thailand/2004	Multiple systems, most organs and tissues
2	A/chicken/Indonesia/7/2003, A/Prachinburi/6231/04	Nervous = cardiovascular = respiratory
3	A/duck meat/Anyang/2001, A/goose/Vietnam/113/2001, A/Hong Kong/213/2003, A/chicken/South Korea/ES/2003	Respiratory >> cardiovascular > skeletal muscle > nervous
4	1997–2001 H5H1 HPAI viruses	No lesions > mild respiratory

vulgaris]), the virus failed to produce evidence of infection or infection was infrequent, and the titers were minimal and without pathological consequence.

Domestic ducks and H5N1 HPAI viruses. However, over the past 9 yr, the H5N1 HPAI virus has evolved into multiple strains that vary in pathogenicity for different bird species. Many of the strains isolated during 1999–2002 from ducks in China when experimentally inoculated into domestic ducks caused infection with shedding from oropharynx and cloaca, but they did not cause illness or death (5). For domestic ducks, using the 2-wk-old duck model and the same intranasal dose of virus (10^6 EID_{50}), new strains have evolved with the ability to produce illness and death along with replicating in internal organs such as the brain (Table 5) (39). Initially (1997–2000), the viruses caused local virus replication in the respiratory tract with associated mild respiratory lesions. However, in 2001, a strain was isolated from frozen duck meat imported from China to South Korea that was able to replicate and spread systemically in domestic ducks without producing clinical signs or death, but virus was isolated from and visualized by immunohistochemistry in meat and brain (43). In 2002–2003, new strains were identified that caused mortality in experimentally inoculated domestic ducks (5,8,18,31). The mortality was the result of systemic infections and was associated with increasing replication titers within respiratory tract (oropharyngeal swabs) and brain (Table 5) compared with nonlethal infections. Pathobiologically, the viruses have changed from being in groups 3 and 4 with increasing severity of lesions in respiratory tract (group 3) to severe lesions in nervous and cardiovascular systems (group 2) to some newer strains producing severe lesions in multiple organs and tissues (group 1) (Table 6). In group 1, the lesions and pathogenesis of the infection and disease are identical between chickens and 2-wk-old ducklings. However, this lethality is age dependent with some strains only causing high mortality in 2- and not 5-wk-old domestic ducks (39).

Other species of birds. Species of wild birds in three orders (Columbiformes, Passeriformes, and Anseriformes) have garnered special interest in the recent H5N1 HPAI outbreaks. Pigeons (order Columbiformes, family Columbidae) have been resistant to infection by most LP and HPAI viruses (14), but there have been reports of pigeon mortality associated with H5N1 HPAI viruses in several countries (9). Initial studies with A/chicken/Hong Kong/220/1997 H5N1 HPAI virus inoculated intranasally into pigeons failed to produce infections (25). Additional studies with two Chinese H5N1 HPAI viruses isolated from chickens during 2004 produced rare infections in intranasally inoculated pigeons and without clinical signs (20). Recently, we conducted additional studies in pigeons by using two different 2004 H5N1 HPAI viruses from Thailand given intranasally (10^6 EID_{50}/bird) (unpublished data). One virus produced illness (4–5 days postinoculation [DPI]) and death of one pigeon (6 DPI) (Table 7). In the other 11 pigeons, six birds had serological or virological evidence of infection, but without clinical disease, whereas the remaining five birds had no evidence of infection. Virologically, the infections were sporadic between 2 and 10 DPI with low titers in oropharynx and cloaca, usually less than 10^3 EID_{50}/ml media. However, the pigeon that died had progressively increasing titers of virus up to $10^{4.4}$ EID_{50}/ml from respiratory (oropharyngeal) and intestinal tract (cloacal) swabs and significant titers in brain ($10^{5.9}$ EID_{50}/g), heart ($10^{4.3}$ EID_{50}/g), kidney ($10^{5.1}$ EID_{50}/g), and lung ($10^{4.7}$ EID_{50}/g). These titers are lower than corresponding samples from chickens that die from H5N1 HPAI viruses (Table 4). The one pigeon that died had severe necrosis of neurons and neuropil in the brain (Fig. 6); moderate necrosis of autonomic neurons with associated lymphocytic ganglioneuritis; severe degeneration and necrosis of cardiac myocytes (Fig. 7) and mild degenerative lesions in skeletal myofibers; mild necrosis of adrenal corticotrophic cells, and rare foci of pancreatic acinar necrosis. Most of the AI virus was localized to neurons and ependymal cells of brain (Fig. 8), autonomic neurons, and cardiac myocytes (Fig. 9), but some antigen was seen in skeletal myofibers, adrenal corticotrophic cells, granulocytes, capillary endothelium, a few pancreatic acinar cells, and smooth muscle in large arteries. In a recent study, after nasal and conjunctival sac inoculation of pigeons with very high dose (10^8 EID_{50}) of A/chicken/Indonesia/2003 (H5N1), neurological disease resulted and three of 14 pigeons died (16). The primary lesions and site of virus localization were in the brain. The remaining nine pigeons did not become ill, but they did seroconvert, indicating infection. However, in contact pigeons did not become infected. This finding suggests that pigeon infections with some H5N1 HPAI strains are possible but that they require high-exposure doses. Thus, it is doubtful that H5N1 HPAI virus will become established in feral pigeon populations.

Table 7. Data from experimental studies in pigeons and American crows after IN inoculation with H5N1 HPAI viruses (10^6 EID_{50}).

Virus	Species	Morbidity (no. sick/no. inoculated)	Mortality (no. dead/no. inoculated)	Seroconversion (no. positive/total)	Virus isolation (no. positive/total)
A/chicken/Hong Kong/220/97	Pigeon	0/6	0/6	ND	0/6
A/crow/Thailand/1C/2004	Pigeon	1/6	1/6	2/5	3/5
A/pigeon/Thailand/1B/2004	Pigeon	0/6	0/6	2/6	3/6
A/crow/Thailand/1C/2004	Crow	2/2	2/2	2/2	2/2

For corvids (e.g., crows and jays), reports of infections and death associated with H5N1 HPAI virus have been reported previously (17). In a study with A/crow/Thailand/1C/2005 (H5N1), American crows (*Corvus brachyrhychos*) inoculated intranasally were depressed and had hunched posture, ruffled feathers, and no appetite. Birds were euthanatized on 8 and 9 DPI because of the severe illness. Both birds had severe lymphocytic-to-heterophilic pancreatitis with necrosis, moderate nonsuppurative encephalitis with neuron necrosis, moderate splenic and hepatic hemachromatosis (Fig. 10), moderate lymphocytic-histiocytic myocarditis with necrosis of cardiac myocytes, multifocal lymphocytic adrenalitis with associated necrosis of corticotrophic cells, transmural lymphocytic typhlitis and serositis, and mild myenteric ganglioneuritis. The AI viral antigen was present in neurons in brain, pancreatic acinar cells, and granulocytes and precursors in bone marrow (Fig. 11). Virus was isolated in low titers from cloaca ($<10^2$ EID_{50}/ml) from 2 to 8 DPI and in moderate titers from oropharynx ($<10^4$ EID_{50}/ml) from 1 to 8 DPI. In a related corvid species, magpies (*Pica pica sericea*), natural cases had severe necrotizing pancreatitis and lymphocytic meningoencephalitis with viral antigen localized to areas with lesions (17).

The numbers of studies that have examined infection in nondomestic ducks are minimal. Recently, a study was completed using A/whooper swan/Mongolia/244/2005 (H5N1) inoculated intranasally (10^6 EID_{50}) into five North American duck species: mallard, northern pintail (*Anas acuta*), blue-winged teal (*Anas crecca*), redhead (*Aythya americana*), and wood duck (*Aix sponsa*) (3). The wood ducks developed clinical signs of cloudy eyes, ruffled feathers, rhythmic dilation and constriction of the pupils, severe weakness, incoordination, tremors and seizures, and two of three birds died. The wood ducks had replication of the HPAI virus and shedding of moderate titers ($10^{4.6}$ EID_{50}/ml) from the oropharynx and cloaca. Histological lesions identified included severe, diffuse neuronal necrosis in the brain, necrotizing pancreatitis and adrenalitis, and multifocal myocardial necrosis. By contrast, the mallard, Northern pintail, blue-winged teal, and redhead ducks had sporadic low-titer infections without clinical disease. Neurological disease and lesions were reported in domestic geese inoculated with 1997 H5N1 HPAI virus (25).

The ability of H5N1 HPAI viruses to infect wild birds is a strain-specific feature within this virus lineage. However, the H5N1 HPAI virus that arose in China in 1996 is unique among all the H5 and H7 HPAI viruses by its wider host susceptibility, including the ability to infect some mammal species.

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